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1. Walton et al., Journal of clinical endocrinology and metabolism. 2001 August. Vol. 86, No. 8, pp. 3675-85.
2. Zarinan et al., Human Reproduction. 2001 august, vol. 16, No. 8, pp. 1611-1618.
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Changes in the biological:immunological ratio of basal and GnRH-releasable FSH during the follicular, pre-ovulatory and luteal phases of the human menstrual cycle

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BACKGROUND: Significant changes in charge isoform distribution of serum FSH occur throughout the human menstrual cycle. In the present study, we analysed the impact of the changing endocrine milieu characteristic of the menstrual cycle on the capability of basal and gonadotrophin-releasing hormone (GnRH)-releasable FSH to trigger intracellular signal transduction via the human FSH receptor. METHODS: Seven normal women underwent blood sampling every 10 min for 10 h during the early follicular phase (FP), pre-ovulatory phase (PO) and mid- to late luteal phase (LP) of the menstrual cycle. Serum from successive samples collected across 2 h intervals containing FSH released under baseline and exogenous GnRH-stimulated conditions was tested for bioactivity employing a homologous in-vitro assay. RESULTS: The biological to immunological (B:I) ratio of basal and GnRH-releasable FSH was significantly ($P < 0.05$) higher at LP (range, 0.83 ± 0.07 to 1.35 ± 0.30) than during the FP (0.43 ± 0.02 to 0.65 ± 0.04) and PO (0.49 ± 0.05 to 0.62 ± 0.06). In all phases, the B:I FSH ratio in baseline samples was similar to those exhibited by samples collected after 10 and 90 μg GnRH administration. CONCLUSIONS: The selective increase in the capability of the admixture of FSH isoforms circulating during the LP to activate the FSH receptor, apparently represents an additional mechanism through which the anterior pituitary may regulate the maturation of those follicles destined to ovulate during the coming cycle.

Key words: follicle-stimulating hormone/FSH biopotency/FSH isoforms/human menstrual cycle

Introduction

FSH is synthesized by the anterior pituitary in multiple molecular forms (Wide, 1985a, 1989; Ulloa-Aguirre *et al.*, 1992b, 1995; Stanton *et al.*, 1992; Simoni *et al.*, 1994). As in other members of the glycoprotein family of hormones, (which also includes thyroid-stimulating hormone, LH and chorionic gonadotrophin), these isoforms differ from each other mainly in their post-translationally modified carbohydrate composition (Wide, 1985b; Green *et al.*, 1986; Baenziger and Green, 1988; Bousfield *et al.*, 1996; Ulloa-Aguirre *et al.*, 1999). In human FSH, this structural heterogeneity depends largely on the amount of terminal sialic acid moieties and, to a lesser extent, sulphate residues added at the end of the oligosaccharide attachments (Baenziger and Green, 1988; Ulloa-Aguirre *et al.*, 1995, 1999). As a consequence of their structural differences, the FSH glycoforms show different biological to immunological potency ratios and metabolic clearance rates; less acidic/sialylated isoforms exhibit higher in-vitro biological to immunological (B:I) activity ratios and shorter plasma half-lives than their more acidic counterparts (Wide, 1986; Ulloa-Aguirre *et al.*, 1992b, 1995, 1999; Cerpa-Poljak *et al.*, 1993; Flack *et al.*, 1994a).

Almost all intrapituitary FSH charge isoforms may be detected in the circulation (Padmanabhan *et al.*, 1988; Wide and Bakos, 1993; Zambrano *et al.*, 1995; Phillips *et al.*, 1997; Anobile *et al.*, 1998). Consequently, target tissues within the gonads are exposed to a mixture of circulating isoforms bearing particular biological features and whose relative abundance varies depending on the endocrine status of the donor at the time of collection of the sample (Ulloa-Aguirre *et al.*, 1995). In fact, several studies have consistently demonstrated the occurrence of significant changes in charge isoform distribution of serum FSH throughout the human menstrual cycle, with greater proportions of less acidic isoforms being released during the mid-cycle than in the early-, midfollicular and luteal phases (Padmanabhan *et al.*, 1988; Wide and Bakos, 1993; Zambrano *et al.*, 1995; Anobile *et al.*, 1998). Although it has been documented that the increased secretion of less acidic/sialylated FSH isoforms at midcycle is temporarily associated with a marked decrease in the plasma half-life of endogenous or exogenous GnRH-provoked serum FSH pulses (Weltman *et al.*, 1994; Zambrano *et al.*, 1995), results from different attempts to document parallel changes in potency of the circulating isoform mixture released throughout the cycle have

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been rather contradictory. Whereas some studies employing heterologous cell systems have detected higher in-vitro B:I FSH activity ratios (a relationship frequently employed as an index of variations in biological activity) in samples obtained during the early follicular phase (Reddi *et al.*, 1990) or mid-follicular phase (Padmanabhan *et al.*, 1988), others have found constant ratios throughout the cycle (Jia *et al.*, 1986; Fauser *et al.*, 1989). Likewise, studies employing homologous assay systems have found higher ratios during the late follicular phase and/or mid- to late luteal phase of the cycle (Gudermann *et al.*, 1994; Christin-Maitre *et al.*, 1996).

In the present study, we attempted to define whether the changing endocrine milieu characteristic of the menstrual cycle modifies the capability of the circulating mixture of FSH isoforms synthesized and secreted by the pituitary gland to trigger intracellular signal transduction at the human FSH receptor level. To accomplish this purpose, we analysed the changes in in-vitro B:I ratio of FSH circulating under baseline conditions as well as those of the admixture of FSH molecules released immediately after administration of submaximal and supraphysiological doses of GnRH at each phase of the menstrual cycle. We reasoned that the consecutive i.v. administration of a low (10 μg) and high (90 μg) GnRH dose would allow the analysis of the changes in potency of the structurally heterogenous population of FSH molecules discharged from an intracellular readily releasable pool as well as those of the molecules that may be newly synthesized under the influence of the particular endocrine milieu prevailing at each given phase of the cycle (Wang *et al.*, 1976a,b).

Materials and methods

Subjects and study design

Twelve normal women, aged 21–27 years, with ovulatory menstrual cycles, as evidenced by midluteal phase serum progesterone concentrations above 19 nmol/l during the recruitment period of the study, agreed to participate. Approval from the human ethics committee of the institute and informed written consent from the volunteers were obtained. All subjects were in good general health and exhibited a body weight within ±10% of ideal body weight. Volunteers had no acne, hirsutism, or galactorrhea, reported regular menstrual cycles of 26–31 days of duration within the previous 5–6 months, and had not received any hormonal treatment during a pre-study follow-up period of at least 3 months. In each woman, the mean duration of the menstrual cycle was estimated from the length of the last 5–6 cycles. On predetermined days of the presumptive early follicular, preovulatory, and mid-luteal phases of the same menstrual cycle, each woman was admitted to the metabolic unit at 0700 h, at which time an indwelling heparinized i.v. catheter was placed in an antecubital vein. Starting at 0800 h, venous blood samples were obtained every 10 min for 10 h; at the beginning of the third hour, subjects received a rapid i.v. bolus of 10 μg synthetic GnRH (Serono de México, S.A. de C.V., Mexico) and, 4 h later, a second GnRH bolus containing 90 μg of the decapeptide. Subjects were recumbent during the study period and were allowed to consume light meals at 10:00 h and 16:00 h. Blood samples were allowed to clot at room temperature for 30 min, then were centrifuged at 1000 *g*. Sera were separated into two aliquots and stored at –20°C until the assay was performed. Samples from five of the volunteers were excluded from the study because the duration of their study cycles was considerably shorter or longer than expected. The remaining seven women were studied on three occasions, once each during the presumptive early follicular phase [FP; days –15 to –10, considering as reference point (day 0) the probable day of ovulation], preovulatory phase (PO; late follicular phase to midcycle; cycle days –3 to 0) and mid- to late luteal phase (LP; days 7–10) of a single cycle. A significant increase in serum progesterone concentrations during the luteal phase (Table I) retrospectively confirmed the occurrence of ovulation in all cycles studied.

Immunoassays of FSH, LH, oestradiol and progesterone

Each subject's set of samples was processed in duplicate for FSH determinations in a single radioimmunoassay (RIA). The FSH RIA was performed employing ^{125}I-labelled FSH I-1 as the tracer (specific activity 60–70 μCi/μg protein), the LER-907 preparation as the standard and anti-human FSH-6 at a final dilution of 1:250 000, as the antiserum (Timossi *et al.*, 1998). This antiserum exhibits <0.1% cross-reactivity with highly purified human LH and prolactin (PRL) and undetectable reactivity with free alpha-subunit and growth hormone (GH). The sensitivity of the assay was 0.24 mIU/tube as expressed in terms of the 2nd International Reference Preparation of human menopausal gonadotrophins (2nd IRP-HMG; 1 mg LER-907 = 53 IU). Baseline samples from each study period were analysed for LH, oestradiol and progesterone content by specific RIAs. The RIA of LH was performed employing ^{125}I-labelled LH-I3 as the tracer (specific activity 70–90 μCi/μg protein), the reference preparation LER-907 as the standard and the antihuman LH-3, at a final dilution of 1:800 000, as the antiserum (Castro-Fernández *et al.*, 2000). Cross-reactivity of this antiserum with highly purified FSH, GH and PRL is <0.2%. The sensitivity of the assay was 0.7 IU/l (2nd IRP-HMG; 1 mg LER-907 = 277 IU). All LH and FSH RIA reagents were generously provided by the National Institute of Diabetes, Digestive and Kidney Diseases (NIDDK, Bethesda, MD, USA), through Dr A.F.Parlow from the National Hormone and Pituitary Program (NHPP, Torrance, CA, USA). In both the FSH and LH RIAs, the intra- and interassay coefficients of variation were determined using multiple replicates (n = 3 per dose) of a serum pool collected from postmenopausal women, assayed at dose levels that displaced the corresponding tracers from the antibody at 10–20%, 45–60% and 75–90% total binding; these coefficients ranged from 4–6 and 6–15% respectively. Serum oestradiol and progesterone were determined by RIA after solvent extraction (recoveries >90% for both sex steroids) using antisera provided by the WHO Matched Reagent Programme (Genève, Switzerland) as previously described (Zambrano *et al.*, 1995). Intra- and interassay coefficients of variation for both assays (at 45–55% total binding) were <5 and <8% respectively.

In-vitro bioassay of FSH

For each individual series of samples, those corresponding to the complete (2 h) baseline period and to the low and high GnRH-stimulated study periods were pooled separately, as shown in Figure 1 (five serum pools per subject; pools 1–5 in Figure 1) and assayed for cAMP production and immunoactive FSH content. The capacity of each pool to provoke cAMP production was tested by a homologous in-vitro bioassay, which employs a human embryonic kidney-derived 293-cell line transfected with the human FSH receptor cDNA as previously reported (Zambrano *et al.*, 1996, 1999). The origin, handling, ligand specificity and biochemical properties of the full-length recombinant human FSH receptor expressed by this cell line have been described elsewhere (Tilly *et al.*, 1992). Briefly, cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Life Technologies, Gaithersburg, MD, USA), pH 7.3, supplemented with

Table I. Mean ± SEM serum oestradiol, progesterone, FSH and LH concentrations at follicular, preovulatory, and luteal phases of the menstrual cycle

Cycle phase	Day of cycle[a]	Baseline serum hormone concentrations			
		Oestradiol (pmol/l)	Progesterone (nmol/l)	FSH (IU/l)	LH (IU/l)
Follicular	−15 to −10	139 ± 7[a]	4 ± 0.3[a]	13 ± 0.4[a]	7 ± 0.2[a]
Preovulatory	−3 to 0	712 ± 51[b]	5 ± 0.3[a]	19 ± 1.2[b]	32 ± 2[b]
Luteal	+7 to +10	521 ± 51[b]	27 ± 3.4[b]	12 ± 0.8[a]	10 ± 0.4[c]

[a]Normalized according to the probable day of ovulation as day 0.
Different superscript letters in each column indicate the existence of significant ($P < 0.05$) differences between cycle phases.

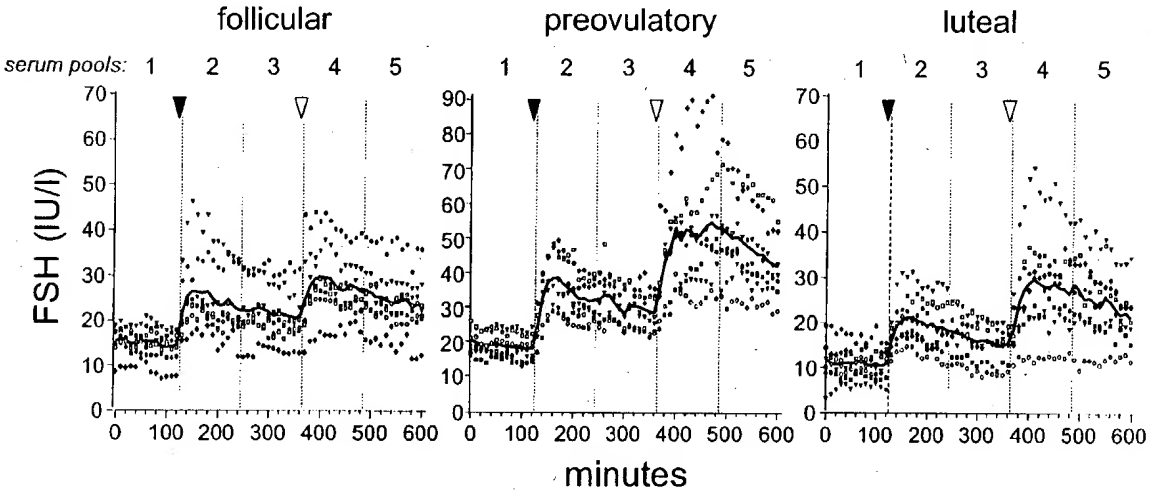

Figure 1. Serum FSH concentration responses to 10 μg (black arrow in each graph) and 90 μg (open arrow in each graph) i.v. injections of exogenous GnRH during the follicular, preovulatory and luteal phases of the menstrual cycle in seven women (symbols). The continuous lines represent the group means. Aliquots of samples from each 120 min study period (delineated by the vertical broken lines) were pooled and the resulting samples (pools 1–5; top of each graph) were analysed for FSH immuno- and in-vitro bioactivity as described in Materials and methods. Note that a different scale is employed in the Y-axis for the pre-ovulatory FSH concentrations.

5% fetal calf serum (FCS), 2 mmol/l L-glutamine, 100 mg/ml geneticin (Life Technologies), 100 IU/ml penicillin and 100 μg/ml streptomycin (Sigma Chemical Co, St Louis, MO, USA) and grown in 162 cm^2 flasks (Costar, Cambridge, MA, USA). Confluent cells were scraped and plated in 24-well culture plates for 24 h at 37°C in 5% CO_2. Cells (5×10^4 cells/culture dish) were then washed and exposed to increasing doses (50–100 μl) of each serum pool or human recombinant FSH (rFSH; Org32489, Organon International BV, Oss, Holland) (1 ng rFSH = 3.29 ng LER-907 according to RIA calibration curves) in the presence of 0.2 mmol/l 1-methyl-3-isobutyl-xanthine (Sigma) dissolved in 400 μl of DMEM supplemented with 2.5% FCS, L-glutamine and antibiotics for 24 h at 37°C. Samples (unknowns and standards) were diluted with serum from women treated with oral contraceptives that contained FSH immuno- and bioactivity not distinguishable from the zero dose, such that the final concentration of human serum in each sample did not exceed 20% (100 μl/culture well). To avoid the interference of serum factors in the bioassay, all serum samples were heated at 56°C for 10 min duration (Gudermann *et al.*, 1994). After incubation, the media and cells were boiled at 95°C for 3 min and stored frozen at −20°C. The minimal detectable response of the assay was 0.25 mIU/well rFSH/tube. All pools from a single subject were bioassayed in triplicate incubations in a single assay run. The inter- and intra-assay coefficients of variation at the effective dose, 50% response (ED50) were <17 and <10% respectively. Aliquots (50–100 μl) of samples from each serum pool series bioassayed were additionally analysed for immunoreactive FSH content by RIA as described above but employing rFSH to construct the standard curve (sensitivity = 0.016 mIU/tube).

cAMP RIA

Total (intra- plus extracellular) cAMP concentrations were determined by RIA after acetylation of the samples and cAMP standards. The RIA of cAMP was performed as previously described (Zambrano *et al.*, 1999) employing 2-O-monosuccinyl cAMP tyrosylmethyl ester (Sigma) iodinated by the chloramine-T method and the CV-27 cAMP antiserum (NIDDK) at a final dilution of 1:75 000. After incubation at 4°C for 24 h, antibody-bound and free cAMP were separated by ethanol precipitation followed by centrifugation at ×1200 *g* at 4°C. The sensitivity of the assay was 4 fmol/tube and the inter- and intra-assay coefficients of variation were <12 and <6% respectively.

The relative in-vitro biological activity of FSH was calculated by interpolation. Data are expressed as the mean B:I activity ratio, the ratio of FSH activity exhibited by serum pools 1–5 in the in-vitro bioassay relative to that yielded by the immunoassay, calculated after conversion of the results to IU/l relative to the 2nd IRP-HMG.

Table II. Areas under the FSH curve and maximal FSH increase after low- and high-dose GnRH administration in normal women during the follicular, preovulatory and luteal phase of a menstrual cycle. Values are the means ± SEM

Cycle Phase	Condition				
	Basal	GnRH 10 μg		GnRH 90 μg	
	aFSHc (IU/l/2 h)	aFSHc (IU/l/4 h)	ΔFSH (IU/l)	aFSHc (IU/l/4 h)	ΔFSH (IU/l)
Follicular	1353 ± 181[a]	4891 ± 725[a,b]	13.6 ± 2.6[a]	6344 ± 709[a]	10.7 ± 1.0[a]
Preovulatory	1506 ± 172[a]	6704 ± 700[a]	21.4 ± 3.0[b]	11194 ± 1374[b*]	32.0 ± 4.6[b*]
Luteal	1098 ± 144[a]	4504 ± 334[b]	12.6 ± 1.7[a]	6909 ± 651[a*]	18.0 ± 3.0[a*]

*$P < 0.05$ versus GnRH 10 μg.
Different superscript letters in each column indicate the existence of significant ($P < 0.05$) differences between cycle phases.

Statistical analysis

The areas under the FSH curve (aFSHc) were calculated by the trapezoid method with the aid of a validated, in-house-built, computer program. Maximal responses to low- and high-dose exogenous GnRH (ΔFSH) was defined as the difference between the maximal FSH concentration and the FSH concentration preceding each GnRH dose (i.e. FSH concentrations at 120 and 360 min). Differences between means of the aFSHc, ΔFSH, B:I FSH ratios and baseline serum oestradiol, progesterone, LH and FSH concentrations within and/or between each cycle phase were analysed by the Bonferroni protected Student's paired two-tailed *t*-test or by ANOVA followed by Student's paired or unpaired *t*-tests as appropriate. Tests for parallelism among the slopes generated by the different serum pools and rFSH were performed following an established method (De Lean *et al.*, 1978). Values are reported as the mean ± SEM, unless specified. Probabilities < 0.05 were considered statistically significant.

Results

Baseline serum gonadotrophin, oestradiol and progesterone levels and FSH response to exogenous GnRH

The baseline serum gonadotrophin and sex steroid hormone levels are shown in Table I. Physiological proximity to the midcycle gonadotrophin surge was corroborated by the significantly elevated baseline concentrations of serum LH and the marked facilitation of the FSH release by exogenous GnRH at this time (Figure 1); likewise, a significant rise in serum progesterone concentrations confirmed the occurrence of ovulation in all studied women. A significant increase in serum FSH concentrations was observed after administration of the two consecutive exogenous GnRH pulses in all cycle phases (Figure 1). Maximal serum FSH concentrations occurred at 40 min (median) (follicular and preovulatory phases) and at 50 min (luteal phase) after the low GnRH dose, and at 40, 60 and 50 min (follicular, preovulatory and luteal phases respectively) after the high GnRH dose. Although baseline aFSHc were similar among the different cycle phases, the magnitude of the FSH response in terms of area and ΔFSH of the GnRH-induced FSH bursts were higher during the preovulatory phase (Table II). During the preovulatory and luteal phases, administration of the 90 μg GnRH dose elicited significantly higher FSH responses than the lower dose, whereas during the early follicular phase, the response to the high-dose GnRH challenge was slightly higher only when the response was expressed in terms of aFSHc.

In-vitro B:I ratio of serum FSH

The five serum FSH pools from each subject (pools 1–5 in Figure 1) were tested at two or three doses for FSH bioactivity using the homologous in-vitro bioassay system. Incubation of HEK-293 cells expressing the rFSH receptor with increasing amounts of rFSH or the serum pools from each set of samples, induced significant and parallel dose-dependent cAMP accumulation (Figure 2). In all cycle phases, the baseline B:I FSH ratios (determined in pool 1) were similar to those exhibited by the pools containing samples obtained after the low- and high-dose GnRH challenges [pools 2 and 3 (collected from samples obtained 10–120 and 130–240 min after low-dose GnRH administration) and pools 4 and 5 (obtained 10–120 and 130–240 min after the high-dose GnRH challenge) respectively] (Table III). The B:I ratio in serum pools collected after administration of the high GnRH dose (pools 4 and 5) were significantly lower than the ratios yielded by pools 2 and 3 only during the early follicular phase. There were no significant differences in B:I FSH ratio between each pair of serum pools collected after administration of 10 and 90 μg exogenous GnRH. In all conditions studied (baseline and low- and high-dose GnRH administration) the B:I FSH ratios were higher (1.5–2.2 fold) during the luteal phase than during follicular phases or preovulatory phases (Table III).

Discussion

Carbohydrates in FSH and other glycoprotein hormones play a major role in structure and function of the molecule. Oligosaccharide attachments influence not only a number of intracellular processes such as intracellular folding of the subunits and secretion of the glycoprotein heterodimer, but also its circulatory survival and capacity to evoke signal transduction at the target cell receptor level (Sairam, 1989; Bishop *et al.*, 1994, 1995; Flack *et al.*, 1994b; Fares *et al.*, 1996; Ulloa-Aguirre *et al.*, 1999). There is compelling evidence that FSH is produced and released in multiple molecular glycoforms (Ulloa-Aguirre *et al.*, 1995; Lambert *et al.*, 1998). Evidence for hormonal regulation of gonadotrophin hetero-

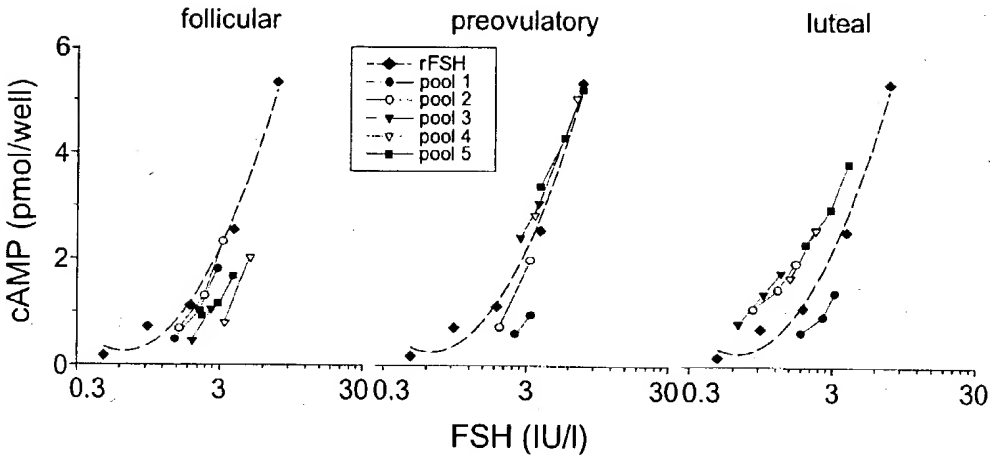

Figure 2. Impact of increasing concentrations of FSH in serum pools 1–5 (symbols) from a woman studied during the follicular, preovulatory and luteal phases of an ovulatory cycle to elicit cAMP production by HEK-293 cells transfected with the full-length rFSH receptor. The dose is expressed in terms of rFSH as measured by RIA of each serum pool (50–100 µl).

Table III. Biological to immunological (B:I) FSH activity ratio in the various serum pools (see Figure 1) from normal women during the follicular, preovulatory and luteal phases of a menstrual cycle. Values are the means ± SEM

Cycle Phase	Serum pool				
	Pool 1	Pool 2	Pool 3	Pool 4	Pool 5
Follicular	0.55 ± 0.06[a]	0.65 ± 0.04[a]	0.62 ± 0.06[a]	0.47 ± 0.04[a*]	0.43 ± 0.02[a**]
Preovulatory	0.49 ± 0.05[a]	0.54 ± 0.02[a]	0.62 ± 0.05[a]	0.53 ± 0.03[a]	0.50 ± 0.05[a]
Luteal	0.83 ± 0.07[b]	1.22 ± 0.20[b]	1.35 ± 0.30[a]	1.09 ± 0.26[b]	0.83 ± 0.13[b]

*$P < 0.05$ versus pool 2.
**$P < 0.05$ versus pool 3.
Different superscript letters in each column indicate the existence of significant ($P < 0.05$) differences between cycle phases.

geneity has been additionally provided by a number of studies in experimental animals and in humans, showing that the relative abundance of the circulating glycoforms changes depending on the endocrine milieu prevailing in the donor subject at the time of study (Padmanabhan *et al.*, 1988; Dharmesh and Baenziger, 1993; Wide and Naessén 1994; Wide *et al.*, 1995; Zambrano *et al.*, 1995; Anobile *et al.*, 1998; Damián-Matsumura *et al.*, 1999).

In the present study, we analysed how variations in relative abundance of the secreted FSH glycoforms occurring under the influence of the changing hormonal milieu characteristic of the menstrual cycle (Padmanabhan *et al.*, 1988; Zambrano *et al.*, 1995; Anobile *et al.*, 1998) may impact the capability of the admixture of circulating FSH isoforms to trigger intracellular signal transduction mediated by the human FSH receptor. The results showed that the B:I ratio of the FSH molecules released either under the endogenous GnRH drive (i.e. in baseline conditions) or after pituitary exposure to supraphysiological doses of GnRH, increased significantly during the luteal phase of the cycle. This finding is in agreement with the results of a previous study in which high B:I FSH ratios were detected in samples collected during the mid- to late luteal phase of normally cycling women as disclosed by an homologous in-vitro bioassay similar to that employed in the present study (Christin-Maitre *et al.*, 1996). The results, however, contrast with some observations derived from the application of heterologous assay systems, in which high B:I ratios were detected in samples collected either during the early or late follicular phase of the cycle (Padmanabhan *et al.*, 1988; Reddi *et al.*, 1990). These apparent discrepancies may be due either to the use of different cell systems (heterologous versus homologous) and/or end points to assess the response, or to differences in sensitivities between the assays. In heterologous cell systems, the response to a given stimulus may be potentially influenced by a number of endocrine, autocrine and paracrine factors, whose impact on the pre-existing level of receptor expression (which is generally lower than in systems stably expressing cloned receptors) and the response measured may be difficult to control, whereas homologous cell assay systems, albeit not provided with all the intracellular machinery that may potentially be involved in alternate signalling pathways secondarily activated as the result of a particular ligand-receptor interaction (Padmanabhan *et al.*, 1991; Sairam *et al.*, 1996; Babu *et al.*, 2000; Gonzalez-Robayna *et al.*, 2000), may bear higher receptor specificity for its cognate ligand (Zambrano *et al.*, 1999). On the other hand, although generation

of cAMP is generally considered as a reliable indicator that activation of the receptor-Gs protein system has occurred (Reichert and Dattatreyamurty, 1989), it represents only an early step in intracellular signal transduction and does not always correlate with a given final cellular response (Padmanabhan *et al.*, 1991; Timossi *et al.*, 2000). However, we have recently observed that excluding the more basic FSH variant [which behaves as an antagonist of FSH action in heterologous but not in homologous in-vitro cell assay systems (Dahl *et al.*, 1988; Timossi *et al.*, 1998)], there is a good correlation at least between the oestrogen and cAMP responses elicited by the pituitary glycoforms in heterologous and homologous cell assays respectively (Zambrano *et al.*, 1999).

The finding that the B:I ratios yielded by pooled samples containing high serum FSH concentrations (as those collected during the exogenous GnRH-provoked FSH surges) were similar to those detected in baseline conditions, does not support the previously suggested possibility that immuno- and/or bioassay sensitivities may underlie the differences in ratios detected across the menstrual cycle (Reddi *et al.*, 1990; Simoni and Nieschlag, 1991; Robertson *et al.*, 1997). Further, although several lines of evidence suggest that differences in glycosylation may affect the reactivity of glycoproteins towards certain antibodies (Papandreou *et al.*, 1990, 1991; Labbe-Jullie *et al.*, 1992; Zerfaoui and Ronin, 1996) and consequently alter the B:I ratio of the hormone, we and others have found that at least for the naturally occurring FSH charge glycoforms, the potential discrepancies among different (polyclonal, monoclonal, and polyclonal/monoclonal) quantitative immunoassays are actually minor and do not significantly alter the calculated in-vitro B:I ratio of the human FSH glycoforms (Zambrano *et al.*, 1996; Oliver *et al.*, 1999).

In view of the several foregoing considerations, we may conclude that the mixture of FSH molecules released from the pituitary during the mid- to late luteal phase of the menstrual cycle contains forms that allow for an overall enhanced capability of the circulating gonadotrophin to trigger signal transduction at the human FSH receptor level. The results of the present study, however, do not allow us to rule out completely the possibility that the increased secretion of less acidic/sialylated FSH isoforms occurring during the late follicular and preovulatory phases of the cycle (Padmanabhan *et al.*, 1988; Zambrano *et al.*, 1995; Anobile *et al.*, 1998) may impact the net response of the naturally expressed target cell to the gonadotrophic stimulus in terms of a given final response such as synthesis of oestradiol or tissue-type plasminogen activator (whose production is important for the proper maturation of the preovulatory follicle and follicular wall rupture) or granulosa cell proliferation (Barrios-De-Tomassi *et al.*, 2001). In this regard, we have recently found that the different glycoforms of FSH may exert differential effects *in vitro*, with the more acidic isoforms exhibiting higher potency to stimulate α-inhibin subunit mRNA expression and the less acidic an enhanced capacity to evoke cytochrome P450 aromatase and tissue-type plasminogen activator mRNA production (Timossi *et al.*, 2000). Whether the increased secretion of more acidic FSH isoforms [with a high capacity to trigger intracellular signal transduction (Christin-Maitre *et al.*, 1996; present study)] during the mid to late luteal phase, contribute to the rise of inhibin-B observed during the luteal-follicular transition (Welt *et al.*, 1997) is an issue that deserves further study. The observation that the charge-based microheterogeneity of circulating FSH is similar in samples taken during the early to mid follicular phase and the luteal phase of the cycle (Padmanabhan *et al.*, 1988; Zambrano *et al.*, 1995), strongly suggests that factors other than terminal sialylation may be involved in the increased capacity of luteal FSH to evoke signal transduction. In this vein, we have recently found that the pituitary FSH charge isoforms contain species with variant carbohydrate chain inner structures that may profoundly influence the capability of the gonadotrophin molecule to trigger a biological response in in-vitro systems (Creus *et al.*, 2001).

The biochemical mechanisms subserving the increased capability of luteal FSH to evoke receptor activation and intracellular signalling are unclear. Exposure of the anterior pituitary to gonadal and/or hypothalamic factors, including luteal steroids, inhibin A and GnRH during this particular cycle phase may be involved in this process. The recognition in experimental animals and in humans that progestins can partially block either the GnRH- or oestradiol-stimulated changes in the charge isoform distribution of FSH secreted *in vitro* or *in vivo* (Ulloa-Aguirre *et al.*, 1992a; Wide *et al.*, 1995), that administration of steroid-free bovine follicular fluid (a source of inhibin) to oophorectomized adult hamsters reduces FSH concentrations and concomitantly the relative abundance of the less acidic/sialylated FSH isoforms (Galle *et al.*, 1983) and that oestrogen administration reduces the activity and mRNA expression of some enzymes involved in terminal sialylation and sulphation of pituitary gonadotrophins (Dharmesh and Baenziger, 1993; Damián-Matsumura *et al.*, 1999; Ulloa-Aguirre *et al.*, 1999) collectively suggest that a plurality of factors participate in the regulation of FSH glycosylation and thus in the ability of the gonadotrophin to induce expression of a measurable biological effect at the target cell level. In this regard, it is interesting to note that acute administration of low or high GnRH doses did not modify the in-vitro B:I ratio of serum FSH during the preovulatory or luteal phases of the cycle, an observation that contrasts with some data showing that this releasing peptide favours the release of bioactive LH molecules *in vivo*, particularly in conditions associated with an oestrogen-enriched hormonal milieu (Veldhuis *et al.*, 1989; Urban *et al.*, 1991). Further, we have previously found that administration of GnRH, at doses similar to those employed in the present study, does not significantly modify the charge isoform distribution of secreted FSH at any cycle phase (Zambrano *et al.*, 1995). Whether GnRH modulates post-translational processing of the FSH oligosaccharides at sites other than terminal sialic acid and/or sulphate residues, is an issue that remains to be investigated more deeply. The present data suggest that whatever the nature of the GnRH-promoted structural modifications introduced, the intrinsic biological activity of the FSH signal to be released from the different functional pools is not modified in a significant manner, at least as revealed by homologous bioassay systems. Although the B:I ratio of FSH in pooled samples collected after administration of 10 μg GnRH during the

early follicular phase did not differ from that exhibited by baseline samples, the administration of a supraphysiological dose (90 μg) of exogenous GnRH was associated with a slight decrease in B:I ratio; this reduction may be secondary to a limited pituitary reserve to synthesize bioactive forms of the gonadotrophin under relatively hypo-oestrogenic, highly demanding conditions (such as that imposed by the pharmacological dose of exogenous GnRH).

In summary, by employing an homologous human FSH receptor in-vitro bioassay system we have observed that, during the mid- to late luteal phase of the menstrual cycle, the anterior pituitary gland releases FSH molecules possessing an increased capability to stimulate their cognate receptor and evoke intracellular signal transduction. This selective increase in the potency of the admixture of FSH isoforms circulating during this particular cycle phase may apparently represent one additional mechanism through which the pituitary gland may regulate the maturation of the cohort of follicles destined to ovulate during the next cycle. Additional studies, that employ naturally occurring homologous cell systems and matrices as well as various cell response parameters, seem necessary for a better understanding of the consequences of physiological changes in the structure of the gonadotrophin signal secreted by the pituitary gland throughout the human menstrual cycle.

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